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VERIFICATION OF TRANSLATION

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declare as follows:

1. That I am well acquainted with both the English and Japanese languages, and
2. That the attached document is a true and correct translation made by me to the best of my knowledge and belief of:-

- (a) Japanese Patent Application No. Hei 7-285417
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(Date)

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This is to certify that the annexed is a true copy of the following application as filed with this Office.

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Application Number: 7-285417

Applicant(s): Dनावेक Research Inc.

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【List of Attached Documents】

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【Name of Document】 Drawings 1

【Name of Document】 Abstract 1

【Proof】 Necessary

[Document Name] Specification

[Title of the Invention] Recombinant Sendai Virus

[Claims]

5 [Claim 1] A recombinant Sendai virus containing a genome having
adesiredforeigngeneinserted, oradesiredgenedeletedorinactivated,
but retaining the disseminative capability.

[Claim 2] The recombinant Sendai virus of Claim 1 in which one
or more than one gene encoding functional proteins are altered.

10 [Claim 3] The recombinant Sendai virus of Claims 1 or 2 comprising
a foreign gene.

[Claim 4] An RNA molecule comprising RNA contained in the
recombinant Sendai virus of any one of Claims 1-3.

[Claim 5] An RNA molecule comprising cRNAs of RNAs contained
in the recombinant Sendai virus of any one of Claims 1-3.

15 [Claim 6] A kit comprising
a. a DNA molecule containing a template cDNA capable of transcribing
RNA of Claims 4 or 5, and
b. a unit capable of transcribing said RNA of Claims 4 or 5 with
said DNA as template in vitro or intracellularly.

20 [Claim 7] A kit comprising
a. a host expressing the NP, P/C and L proteins of Sendai virus (each
protein may be replaced with a protein of an equivalent activity),
and
b. the RNA molecule of Claims 4 or 5.

25 [Claim 8] A method for producing the recombinant Sendai virus
of any one of Claims 1-3, comprising transfecting RNA of Claims 4
or 5 to a host expressing the NP, P/C and L proteins of Sendai virus
(eachproteinmaybereplacedwithaproteinofanequivalentactivity).

[Claim 9] A kit consisting of the following three components,
30 a. a host expressing the NP, P/C and L proteins of Sendai virus,
b. a DNA molecule containing a template cDNA capable of transcribing
RNA or cRNA of any one of Claims 4 or 5, and
c. a unit capable of transcribing RNA of Claims 4 or 5 with said
DNA as template in vitro or intracellularly.

35 [Claim 10] A method for producing the recombinant Sendai virus
of any one of Claims 1-3, wherein said method comprises introducing

a DNA molecule containing a template cDNA capable of transcribing RNA of Claims 4 or 5, and a unit capable of transcribing RNA of Claims 4 or 5 with said DNA as template in vitro or intracellularly into a host expressing the NP, P/C and L proteins of Sendai virus.

5 [Detailed Description of the Invention]

[0001]

[Technical Field of Industrial Application]

The present invention relates to the recombinant Sendai virus and the method for preparing the same.

10 [0002]

[Prior Art]

Sendai virus is also named hemagglutinating virus of Japan (HVJ), and classified in parainfluenza virus type I, belonging to the genus Paramyxovirus of the family Paramyxoviridae.

15 [0003]

Sendai virus particle is pleomorphic, having the genome RNA without a function as template for translation (hereafter designated "negative strand RNA") enclosed in an envelope of 150-200 nm in diameter.

20 Historically, Sendai virus has also been regarded as an industrially useful virus, being widely utilized, especially for the production of heterokaryons and hybrid cells, by taking advantage of viral cell-fusion capacity. Also, cell-fusing liposomes have been developed as a vehicle for gene therapy. Furthermore, Sendai virus is also used as the inducer for various interferons.

25 [0004]

According to the classification based on the karyotype of genome nucleic acid, Sendai virus belongs to a group of negative single-strand RNA viruses of negative strand RNA viruses among RNA viruses. RNA viruses are classified into three groups, the dsRNA viruses (double stranded RNA viruses), positive strand RNA viruses, and negative strand RNA viruses. The dsRNA virus group includes reovirus, rotavirus, phytoreovirus, etc., and have segmented, plural filamentous dsRNA genome. Positive strand RNA viruses include poliovirus, Sindbis virus, Semliki forest virus, and Japanese B encephalitis virus, which
35 possess a single positive sense RNA as genome. The genome RNA can function as an mRNA and is capable of producing proteins required

for RNA replication and particle formation depending on the translational functions of host cells. In other words, the genome RNA itself of positive strand RNA viruses is capable of disseminating.

In the present specification, by "disseminative capability" is meant "the capability to form infectious particles or their equivalent complexes and successively disseminate them to other cells following the transfer of nucleic acid into host cells by infection or artificial techniques and the intracellular replication of said nucleic acid.

Sindbis virus classified to positive strand RNA viruses and Sendai virus classified to negative strand RNA viruses have both infectivity and disseminative capability. On the other hand, adeno-associated virus classified to the parvovirus family has the infectivity but no disseminative capability (the mixed infection of adenovirus is necessary for the formation of viral particles). Furthermore, the positive strand RNA derived from Sindbis virus which is artificially transcribed *in vitro* is disseminative (to form infectious viral particles when transfected into cells). In contrast, not only negative strand but also positive strand of Sendai viral RNA artificially transcribed *in vitro* is not disseminative (form no infectious viral particles when transfected into cells).

[0005]

Recently, viral vectors have been used as vehicles for gene therapy. In order to use them as gene therapy vectors, it is necessary to establish techniques for reconstituting viral particles. (By "reconstitution of viral particles" is meant the artificial formation of viral genome nucleic acid and the production of original or recombinant viruses *in vitro* or intracellularly.) This is because, in order to transfer foreign genes into viral vectors, viral particles should be reconstituted from the viral genome with foreign genes integrated by the gene manipulation. Once techniques of viral reconstitution are established, it becomes possible to produce viruses with a desired foreign gene introduced, or with desired genes deleted or inactivated.

[0006]

Also, once the viral reconstitution system is constructed and the viral gene manipulation becomes possible, said system appears

to become a potential tool for genetically analyzing the viral function.

Genetic analysis of viral functions is very important from the medical viewpoint of prevention and therapy of diseases etc. For example, if the replication mechanism of viral nucleic acid is elucidated by utilizing the differences between viral metabolism and host-cellular metabolism, it may be possible to develop viricide acting on the viral nucleic replication process and less damaging to host cells. Also, by elucidating functions of viral gene-encoded proteins, it may become possible to develop antiviral drugs targeting proteins related with the viral infectivity and particle formation.

Furthermore, by modifying genes concerned with the membrane fusion and preparing liposomes with superior membrane-fusing capability, it will be able to use them as gene therapy vectors. In addition, as represented by the interferon, the viral infection may induce the activation of host genes for viral resistance, resulting in the enhanced viral resistance of hosts. Genetic analysis of virus functions may provide more important information on the activation of host genes.

[0007]

Reconstitution of DNA viruses possessing DNA as the genomic nucleic acid has been performed for some time, and can be carried out by the introduction of the purified genome itself, such as SV40, into monkey cells [J. Exp. Cell Res., 43, 415-425 (1983)].

[0008]

Reconstitution of RNA viruses containing an RNA genome has been preceded by positive strand RNA viruses since genomic RNAs also function as mRNA. For example, in the case of poliovirus, the disseminative capability of the purified genomic RNA itself was already demonstrated in 1959 [Journal of Experimental Medicine, 110, 65-89 (1959)]. Then, it was achieved to reconstitute Semliki forest virus (SFV) by the introduction of cloned cDNAs into host cells utilizing DNA-dependent RNA polymerase activity of host cells [Journal of Virology, 65, 4107-4113 (1991)].

[0009]

Furthermore, using these viral reconstitution techniques, gene therapy vectors have been developed [Bio/Technology, 11, 916-920

(1993); Nucleic Acids Research, 23, 1495-1501 (1995); Human Gene Therapy, 6, 1161-1167 (1995); Methods in Cell Biology, 43, 43-53 (1994); Methods in Cell Biology, 43, 55-78 (1994)].

[0010]

5 However, as described above, in spite of many advantages of Sendai virus to be industrially useful virus, its reconstitution system has not been established, because it is a negative-strand RNA. This is due to tremendous difficulty in reconstituting viral particles via viral cloned cDNAs.

10 [0011]

As described above, it has been clearly demonstrated that a mere introduction of RNA from negative-strand RNA virus (vRNA) or its complementary strand RNA (cRNA) into host cells does not support the generation of negative-strand RNA virus. This is absolutely
15 different from the case of positive strand RNA viruses. Although, in Tokkai H4-211377, "methods for preparing cDNAs corresponding to negative strand RNA viral genome and infectious negative strand RNA virus" are disclosed, the entire experiments of said documents described in "EMBO. J., 9, 379-384 (1990) were later found to be
20 not reproducible, so that the authors themselves had to withdraw all the article contents [see EMBO J., 10, 3558 (1991)]. Therefore, it is obvious that techniques described in Tokkai H4-211377 do not correspond to the related art of the present invention.

[0012]

25 Reconstitution systems of negative strand RNA viruses were reported for influenza virus [Annu. Rev. Microbiol., 47, 765-790 (1993); Curr. Opin. Genet. Dev., 2, 77-81 (1992)]. Influenza virus is a negative strand RNA virus having an eight-segmented genome. According to these literatures, a foreign gene was first inserted
30 into the cDNA of one of said genome segments, and then RNA transcribed from the cDNA of all eight segments containing the foreign gene was assembled with the virus-derived NP protein to form RNP. Then, the virus reconstitution was achieved by providing host cells with these RNPs and an RNA-dependent RNA polymerase. Thereafter, the
35 reconstitution of negative single-stranded RNA virus from cDNA was reported for rabies virus belonging to the rhabdovirus family [J.

Virol., 68, 713-719 (1994)].

[0013]

Therefore, although techniques for reconstituting negative strand viruses have become fundamentally known to the public, in the case of Sendai virus, the direct application of these techniques did not support the viral reconstitution. Also, the reconstitution of viral particles reported on the rhabdovirus was confirmed only by the expression of marker genes, RT-PCR, etc. Furthermore, the yield was not satisfactory for practical applications. Besides, in order to provide factors required for the viral reconstitution within host cells, helper viruses such as wild type viruses, recombinant vaccinia virus, etc. were conventionally introduced to host cells together with nucleic acids of the virus to be reconstituted. Accordingly, difficulties in separating the reconstituted desired virus from these harmful viruses were posing a difficult problem.

[0014]

[Problems to Be Solved by the Invention]

The purpose of the present invention is to establish an efficient system for reconstituting Sendai virus, enabling the gene manipulation of Sendai virus, and providing Sendai viral vector sufficiently useful in the field of gene therapy, etc.

[0015]

[Means to Solve the Problems]

In order to apply to the reconstitution test of Sendai virus, the present inventors first made various investigations using cDNAs derived from Sendai viral DI particles [ref. defective interfering particle in EMBO J., 10, 3079-3085 (1991)] or the minigenome of Sendai virus. As a result, they found efficient conditions regarding weight ratios among materials to be introduced into host cells, including cDNA, cDNAs concerning the transcription and replication, and the recombinant vaccinia virus to provide the T7 RNA polymerase expression unit. Furthermore, the present inventors obtained full-length cDNAs of both positive and negative strands, constructed plasmids to induce the intracellular biosynthesis of positive or negative strand RNA of Sendai virus, and transferred said plasmid into host cells expressing cDNAs concerning the transcription and replication. As a result,

they succeeded in re-constituting Sendai virus particles from cDNAs thereof. The present inventors also found for the first time that cDNAs introduced into host cells are more preferable in the circular form than in the linear form for the efficient reconstitution of viral particles, and that positive strand RNAs are superior to negative strand RNAs in the intracellular transcription for the highly successful reconstitution of viral particles.

[0016]

In addition, the present inventors found that Sendai virus could be reconstituted even without using recombinant vaccinia virus as the T7RNA polymerase expression unit. That is, when the full-length RNA of Sendai virus transcribed *in vitro* was transferred into cells, and cDNAs encoding enzymes for the initial transcription and replication were transcribed under the control of T7 promoter, viral particles were reconstituted. This indicates that, if cells which express all enzymes required for the initial transcription and replication are constituted, the recombinant Sendai virus can be produced entirely without using helper viruses such as vaccinia virus.

Since cells expressing all enzymes required for the initial transcription and replication were already described [J. Virology, 68, 8413-8417 (1994)], those skilled in the art will be able to form such cells with reference to said article. The cell described in said reference is the one derived from the 293 cell line carrying three out of Sendai viral genes, namely NP, P/C and L on its chromosome, and expressing proteins encoded by these three genes, NP, P/C and L.

[0017]

From numerous examples of viral vectors, if viral particles can be efficiently reconstructed from nucleic acids, it is obvious that those skilled in the art are able to readily exchange desired viral gene, insert a foreign gene, or inactivate or delete a desired viral gene. That is, it will be obvious to those skilled in the art that the first success in reconstituting Sendai viral particles by the present invention has enabled the gene manipulation of Sendai virus.

[0018]

That is, the present invention comprises the followings.

1. A recombinant Sendai virus having the genome with a desired foreign gene inserted or a desired gene deleted or inactivated, and retaining the disseminative capability.

2. The recombinant Sendai virus of description 1, wherein more than one gene encoding functional proteins are modified.

3. The recombinant Sendai virus of descriptions 1 or 2 comprising a foreign gene.

4. An RNA molecule comprising RNAs contained in the recombinant Sendai viruses of any one of descriptions 1-3.

5. An RNA molecule comprising cRNAs of RNAs contained in the recombinant Sendai viruses of any one of descriptions 1- 3.

6. A kit consisting of the following two components.

a. a DNA molecule comprising a template cDNA which can transcribe RNAs of descriptions 4 or 5, and

b. a unit capable of transcribing RNAs of descriptions 4 or 5 with said DNA as template in vitro or intracellular.

7. A kit consisting of the following two components.

a. a host expressing the NP, P/C and L proteins of Sendai virus (each protein may be replaced with a protein having an equivalent activity), and

b. an RNA molecule of descriptions 4 or 5.

8. A method for producing the recombinant Sendai virus of descriptions 1-3, comprising introducing the RNA molecule of descriptions 4 or 5 into host cells expressing the NP, P/C and L proteins of Sendai virus (each protein may be replaced by a protein having the equivalent activity).

9. A kit consisting of the following three components,

a. a host expressing the NP, P/C and L proteins of Sendai virus,

b. a DNA molecule comprising a template cDNA capable of transcribing RNAs or cRNAs of descriptions 4 or 5, and

c. a unit capable of transcribing RNAs of descriptions 4 or 5 with said DNA as template in vitro or intracellularly.

10. A method for producing the recombinant Sendai virus of descriptions 1-3, comprising introducing the DNA molecule comprising

a template cDNA capable of transcribing RNAs of descriptions 4 or 5, and a unit capable of transcribing RNAs of descriptions 4 or 5 with said DNA as template *in vitro* or intracellularly into hosts expressing the NP, P/C and L proteins of Sendai virus.

5 [0019]

Recombinant Sendai viral vectors of the present invention can be obtained, for example, by *in vitro* transcribing the recombinant cDNA encoding the gene-technologically produced recombinant Sendai viral vector genome, producing the recombinant Sendai viral genome
10 RNA, and introducing said RNA to a host simultaneously expressing the NP, P/C and L proteins (each protein may be a protein with an equivalent activity) of Sendai virus. Alternatively, Sendai viral vectors of the present invention can be obtained by introducing
a) the recombinant cDNA coding for the gene-technologically produced
15 recombinant Sendai viral vector genome, and
b) a unit capable of intracellularly transcribing RNA with said DNA as template
into a host simultaneously expressing the NP, P/C and L proteins (each protein may be a protein having an equivalent activity) of
20 Sendai virus. In this case, said recombinant cDNA a) may be inserted downstream of a specific promoter, and said transcription unit b) may be a DNA molecule expressing a DNA-dependent RNA polymerase acting on said specific promoter.

[0020]

25 [Mode for Carrying out the Invention]

Sendai virus, the starting material in the present invention for the insertion of a desired foreign gene, or the deletion or inactivation of a desired gene may be a strain classified to parainfluenza virus type I, exemplified by Sendai virus Z strain
30 or Fushimi strain. Furthermore, incomplete viruses such as DI particles, synthetic oligonucleotides, etc. may be used partial materials.

[0021]

So far as the recombinant Sendai virus of the present invention
35 maintain the disseminative capability, any foreign gene may be inserted, and any genome gene may be deleted or modified. Foreign genes to

be inserted may be exemplified by genes encoding various cytokines and peptide hormones. Also, for example, in order to inactivate genes for immunogenicity, or enhance the efficiency of RNA transcription and replication, part of genes related with RNA replication of Sendai virus may be modified. Concretely, for example, at least one of the replication factors, the NP, P/C and L proteins may be modified to enhance or reduce the transcription and replication capabilities. The HN protein, one of the constitutional proteins, has dual activities as hemagglutinin and neuraminidase. For example, the reduction of the former activity may increase the viral stability in blood stream, and the modification of the latter activity may enable the regulation of viral infectivity. Also, the modification of the F protein mediating membrane fusion may be useful for improving membrane fusion liposomes constructed by fusing the reconstituted Sendai virus and artificial liposomes enclosing a desired drug or gene.

[0022]

The present invention has enabled the introduction of point mutation and insertion at any sites of the genomic RNA, and is highly expected to accelerate the accumulation of genetic information on viral functions. For example, once the mechanism of viral RNA replication is elucidated, it may become possible to develop a viricide less harmful to a host cell and targeting nucleic acid replication process by utilizing the differences between the viral and host-cellular metabolisms of nucleic acid. In addition, the elucidation of functions of viral gene-encoded proteins may contribute to the development of viricides targeting proteins involved in infectivity and forming capability of viral particles. Concretely, for example, these techniques may be used for the analysis of antigen-presenting epitopes of the F and HN proteins which may act as antigenic molecules on the cell surface. Also, when a host cell gene for viral resistance is activated by viral infection, resulting in an elevated viral resistance, important information on such activation mechanism of host gene may be obtained by the genetic analysis of viral functions. Since Sendai virus is effective in inducing interferons, it is used in various basic studies. By

analyzing the genome region necessary for inducing interferons, it may be possible to produce a non-viral interferon inducer. Techniques of the present invention are useful for the development of vaccines.

Live vaccines may be produced by inoculating the recombinant Sendai virus with attenuating mutations to embryonated chicken eggs. Information thus obtained may be applied to other negative strand viruses, such as measles virus and mumps virus, with high demand for live vaccines. Furthermore, the present invention has enabled the usage of the recombinant Sendai virus as vectors for gene therapy.

Since virus vectors of the present invention derived from Sendai virus are highly safe in the clinical application and disseminative, and expected to be therapeutically effective with a relatively small dosage. In addition, when the suppression of a viral vector replication becomes necessary at the completion of therapy or during the therapy, only the viral vector replication can be specifically suppressed without damaging hosts by administering an inhibitor of RNA-dependent RNA polymerase.

[0023]

[Examples]

In the following, the present invention will be concretely described with reference to examples, but is not limited to these examples.

[0024]

[Example 1] Preparation of Sendai virus transcription units pUC18/T7(-)HVJRz.DNA and pUC18/T7(+)HVJRz.DNA

Plasmid pUC18/T7(-)HVJRz.DNA was constructed by inserting a DNA molecule comprising T7 RNA polymerase promoter, Sendai virus cDNA designed to be transcribed to the negative strand RNA and the ribozyme gene in this order into pUC18 vector. Also, plasmid pUC18/T7(+)HVJRz.DNA was constructed by inserting a DNA molecule comprising T7 RNA polymerase promoter, Sendai virus cDNA designed to be transcribed to the positive strand RNA and the ribozyme gene in this order into pUC18 vector. Constructions of pUC18/T7(-)HVJRz.DNA and pUC18/T7(+)HVJRz.DNA are shown in Figs. 1 and 2, respectively.

[0025]

[Example 2] Reconstitution experiment of Sendai virus from cDNA

LLC-MK2 cells (2×10^6) trypsinized in a usual manner were placed in a 60-mm diameter plastic dish, and incubated in MEM medium (MEM supplemented with 10% FBS) (2 ml) in a 5% CO₂ atmosphere at 37°C for 24 h. After removing the medium and washing with PBS (1 ml), a suspension of recombinant vaccinia virus vTF7-3 expressing T7 polymerase in PBS (0.1 ml) was added to the cells at the multiplicity of infection (moi) of 2. The dish was gently agitated every 15 min to thoroughly spread the viral solution for 1 h infection. After removing the viral solution and washing with PBS (1 ml), a medium containing cDNA, which was prepared as follows, was added to the dish.

[0026]

Nucleic acids shown in Tables (containing plasmids expressing factors required for the replication of Sendai virus, pGEM-L, pGEM-P/C and pGEM-NP) were placed in a 1.5-ml sampling tube, and adjusted to a total volume of 0.1 ml with HBS (Hepes buffered saline; 20 mM Hepes pH 7.4 containing 150 mM NaCl). In those tables, (-) and (+) cDNAs represent plasmids pUC18/T7(-)HVJRz.DNA and pUC18/T7(+)HVJRz.DNA, respectively, and /C and /L indicate that cDNA is introduced into cells in the circular form and linear form after the treatment with restriction enzyme MluI, respectively.

[0027]

On the other hand, in a polystyrene tube were placed HBS (0.07 ml), DOTAP (Boehringer Mannheim) (0.03 ml). To this tube was added the nucleic acid solution described above, and the mixture was left standing as such for 10 min. Then, to this mixture was added the cell culture medium described above (2 ml, MEM supplemented with 10% FBS) followed by the vaccinia virus inhibitors, rifampicin and cytosine arabinoside C (C/Ara/C), to the final concentrations of 0.1 mg/ml and 0.04 mg/ml, respectively, resulting in the preparation of the medium containing cDNA described above.

[0028]

The dish described above was incubated in a 5% CO₂ atmosphere at 37°C for 40 h. The cells in the dish were harvested using a rubber policeman, transferred to an Eppendorf tube, sedimented by

centrifuging at 6,000 rpm for 5 min, and re-suspended in PBS (1 ml).

Aliquots of this cell suspension, as such or after diluted, were inoculated to 10-days old developing embryonated chicken eggs. That is, the cell suspension was diluted with PBS to the cell numbers shown in Table 1, and eggs inoculated with its 0.5-ml aliquots were incubated at 35°C for 72 h, then at 4°C overnight. Chorio-allantoic fluid was recovered as virus solution from these eggs using a syringe with a needle.

[0029]

Hemagglutinin unit (HAU) and plaque forming unit (PFU) of the recovered virus solution were assayed as follows.

[0030]

HAU was determined as follows. Chicken blood was centrifuged at 400 x g for 10 min and the supernatant was discarded. Precipitates thus obtained were suspended in 100 volumes of PBS, and centrifuged at 400 x g for 10 min to discard the supernatant. This procedure was repeated twice to prepare an 0.1% blood cell solution. Two-fold serial dilutions of virus solutions were prepared, and 0.05 ml each dilution to be assayed was dispensed into each well of 96-well titer plate. The blood cell solution (0.05 ml each) was further added to each well, gently swirled to ensure a thorough mixing, and left at 4°C for 40 min. The highest virus dilution to cause the hemagglutination observable with the naked eye was taken as HAU.

[0031]

PFU was assayed as follows. CV-1 cells were grown to a monolayer on a 6-well culture plate. After the culture medium was discarded, a virus solution 10-fold serially diluted (0.1 ml each) was dispensed into each well of the culture plate to infect the cells at 37°C for 1 h. During the infection, a mixture of 2 x MEM free of serum and melted 2% agar (55°C) was prepared, and trypsin was added to the mixture to a final concentration of 0.0075 mg/ml. After 1 h infection and removal of the virus solution, the culture medium mixed with agar (3 ml each) was added to each well of the culture plate, and incubated under a 5% CO₂ atmosphere at 37°C for 3 days. Phenol red (0.1%) (0.2 ml) was added to each well, incubated at 37°C for 3 h, and then removed. Unstained plaques were counted to estimate the

virus titer as PFU/ml.

[0032]

Table 1 shows Sendai virus template cDNAs transfected into LLC-2 cells, amounts of cDNA factors, pGEM-L, pGEM-P/C, and pGEM-NP, required for the RNA replication, incubation time, cell numbers inoculated to chicken eggs, HAU and PFU values.

[0033]

[Table 1]

Template cDNA	Total amount (μ g)	pGEM -L (μ g)	pGEM -P/C (μ g)	pGEM -NP (μ g)	Incubation time (h)	Amount of cells	HAU	PFU
(+) cDNA/C	10	4	2	4	40	1.00×10^5	512	2×10^9
(+) cDNA/C	10	4	2	4	40	1.00×10^5	256	9×10^8
(+) cDNA/C	10	4	2	4	40	1.00×10^6	256	9×10^8
(+) cDNA/L	10	4	2	4	40	1.00×10^5	<2	<10
(+) cDNA/L	10	4	2	4	40	1.00×10^5	<2	<10
(+) cDNA/L	10	4	2	4	40	1.00×10^6	<2	<10
(-) cDNA/L	10	4	2	4	40	1.00×10^4	<2	<10
(-) cDNA/L	10	4	2	4	40	1.00×10^5	<2	<10
(-) cDNA/L	10	4	2	4	40	1.00×10^6	<2	<10
(-) cDNA/C	10	4	2	4	40	1.00×10^4	<2	<10
(-) cDNA/C	10	4	2	4	40	1.00×10^5	<2	<10
(-) cDNA/C	10	4	2	4	40	1.00×10^6	4	8×10^3

Samples showing both HAU and PFU were sedimented by ultra-centrifugation, re-suspended, purified by a sucrose density gradient centrifugation from 20% to 60%, and fractionated by 12.5% SDS-PAGE. Each protein contained in these samples was the same in size as that of Sendai virus.

[0034]

These results demonstrated that Sendai virus can be reconstituted by introducing cDNAs into cells, and that virus particles are more efficiently reconstituted by introducing cDNAs transcribing positive strand RNAs as compared with those transcribing negative strand RNAs,

and further by introducing cDNAs in the circular form rather in the linear form.

[0035]

[Example 3] Survey of RNA replication factors required for Sendai virus reconstitution

Experiments were performed to examine whether all three plasmids expressing the L, P/C and NP proteins were required for the reconstitution of Sendai virus. Experimental methods were similar to those described in Example 2 except that any combinations of two out of pGEM-L, pGEM-P/C and pGEM-NP plasmids or only one out of them, instead of all these three combined as in Example 2, were introduced together with a template cDNA into cells.

[0036]

Table 2 shows Sendai virus template cDNAs introduced into LLC-MK2 cells, amounts of the cDNA factors required for RNA replication including pGEM-L, pGEM-P/C and pGEM-NP, incubation time, number of cells inoculated into chicken eggs, and values of HAU and PFU.

[0037]

[Table 2]

Template cDNA	Total amount (μ g)	pGEM -L	pGEM -P/C	pGEM -NP	Incubation time (h)	Number of cells inoculated	HAU	PFU
(+) cDNA/C	10	4	2	4	40	1.00×10^5	256	6×10^8
(+) cDNA/C	10	4	2	4	40	1.00×10^6	512	4×10^9
(+) cDNA/C	10	0	2	4	40	1.00×10^6	<2	<10
(+) cDNA/C	10	0	2	4	40	1.00×10^6	<2	<10
(+) cDNA/C	10	4	0	4	40	1.00×10^6	<2	<10
(+) cDNA/C	10	4	0	4	40	1.00×10^6	<2	<10
(+) cDNA/C	10	4	2	0	40	1.00×10^6	<2	<10
(+) cDNA/C	10	4	2	0	40	1.00×10^6	<2	<10
(+) cDNA/C	10	0	0	4	40	1.00×10^6	<2	<10
(+) cDNA	10	0	0	4	40	1.00×10^6	<2	<10
(+) cDNA/C	10	0	2	0	40	1.00×10^6	<2	<10
(+) cDNA/c	10	0	2	0	40	1.00×10^6	<2	<10
(+) cDNA/C	10	4	0	0	40	1.00×10^6	<2	<10
(+) cDNA/C	10	4	0	0	40	1.00×10^6	<2	<10

As shown in Table 2, no virus reconstitution was observed by introducing any combinations of two out of these three factors into cells, confirming the necessity of all three proteins L, P/C and NP for the virus reconstitution.

[0038]

[Example 4] Reconstitution experiment of Sendai virus *in vitro* from transcribed RNAs

Since the reconstitution of Sendai virus from the functional cDNA clones was described in Example 2, it was further examined whether transcription products of said cDNAs *in vitro*, that is, vRNA

and cRNA, can support similar reconstitution.

[0039]

After the Sendai virus transcription units, pUC18/T7(-)HVJRz.DNA and pUC18/T7(+)HVJRz.DNA, were linearized with restriction enzyme MluI, using these DNAs as templates, RNA synthesis was performed *in vitro* with a purified T7 polymerase preparation (EPICENTRE TECHNOLOGIES: Ampliscribe T7 Transcription Kit). The method for synthesizing *in vitro* RNAs essentially followed the protocols provided with the kit. Using RNA products thus obtained in place of cDNAs in Example 2, similar experiments were performed, and the virus production was estimated by HA test.

[0040]

Results are shown in Table 3.

[0041]

15 [Table 3]

Template cDNA	Total amount (μ g)	pGEM-L (μ g)	pGEM-P/C (μ g)	pGEM-NP (μ g)	Incubation time (h)	Number of cells inoculated	HAU	PFU
<i>in vitro</i> (-) RNA	10	4	2	4	40	1.00E+06	512	2×10^9
<i>in vitro</i> (-) RNA	10	4	2	4	40	1.00E+06	512	ND
<i>in vitro</i> (+) RNA	10	4	2	4	40	1.00E+06	2	5×10^3
<i>in vitro</i> (+) RNA	10	4	2	4	40	1.00E+06	<2	ND

These results indicate that virus can be reconstituted by introducing either negative or positive sense strand RNAs into cells.

[0042]

[Effects of the Invention]

By the present invention, a system for efficient reconstitution of viral particles from Sendai viral cDNAs has been established, enabling the gene manipulation of Sendai virus to produce the recombinant Sendai virus comprising a genome with a desired foreign gene inserted or a desired gene deleted or inactivated, but retaining

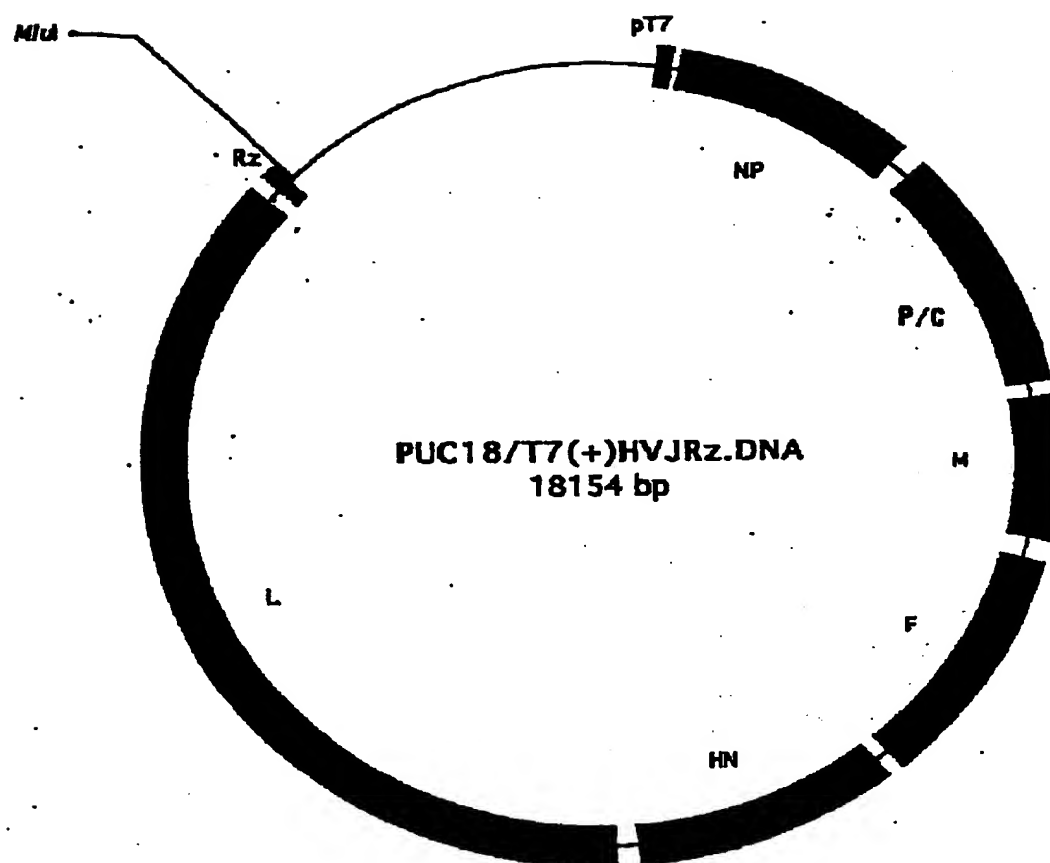
the disseminative capability.

[Brief Description of the Drawings]

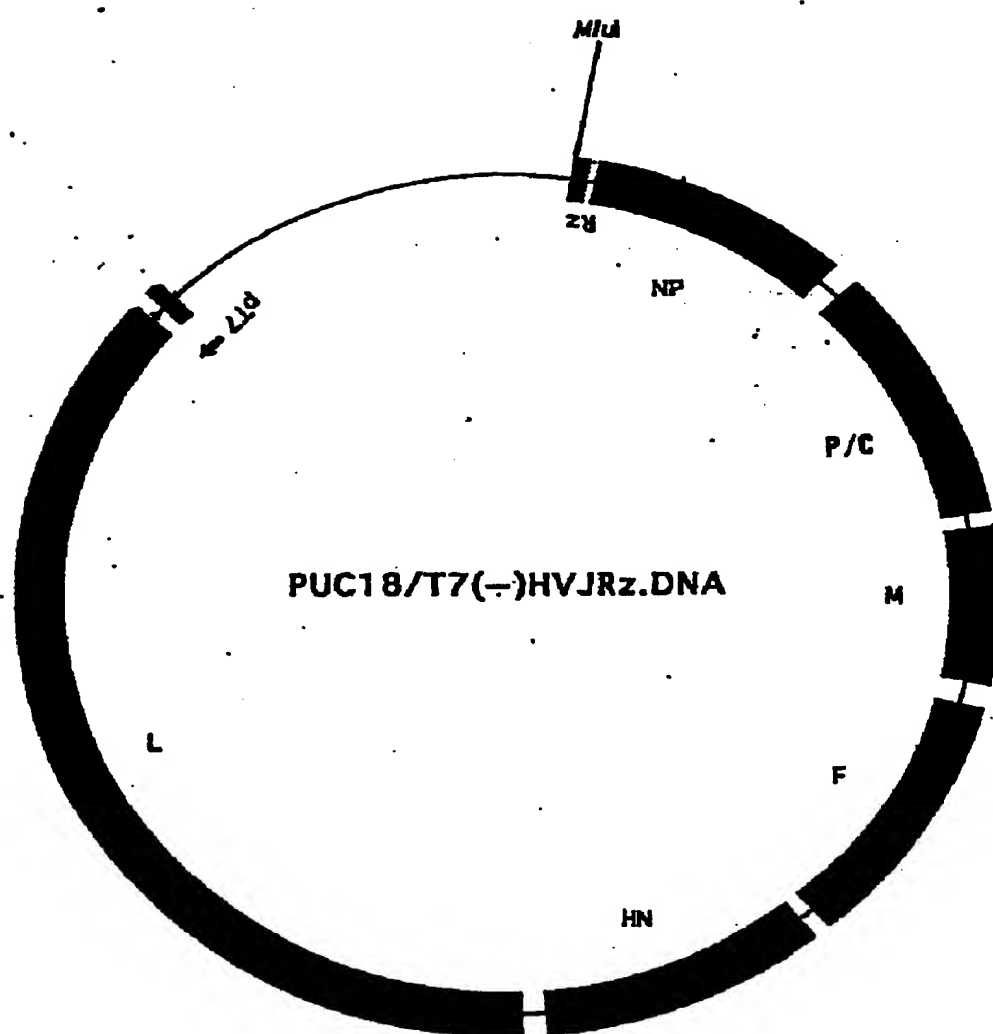
Figure 1 is a schematic representation of plasmid pUC18/T7(-)HVJRz.DNA.

5 Figure 2 is a schematic representation of plasmid pUC18/T7(+)HVJRz.DNA.

[Document Name] Drawings
[Figure 1]



[Figure 2]



[Document Name] ABSTRACT

[Abstract]

[Problems to Be Solved] An objective of the present invention is to develop a method of efficiently producing a highly safe Sendai virus vector and Sendai virus vectors being practical.

[Means to Solve the Problems] A method for regenerating Sendai virus particles by transfecting the Sendai virus genome to a host expressing all genes for the initial viral replication has been developed, enabling the genetic manipulation of Sendai virus and effective utilization of said virus as the vector.

[Selected Drawing] None